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Peroxisome Proliferator-Activated Receptor Isoform Expression Changes in Human Gestational Tissues with Labor at Term

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that are involved in lipid metabolism, differentiation, proliferation, cell death, and inflammation. Three subtypes have been identified: PPAR-α, -δ, and -γ. We have previously shown presence of PPAR-γ mRNA in the amnion, choriodecidua, and placenta, and its level of expression was unchanged with labor. To evaluate whether PPAR-α and -δ subtypes are present in intrauterine tissues, placentae were obtained from women at term after spontaneous vaginal delivery (TSL; $n = 15$) and elective caesarean section before labor (TNL; $n = 15$). Northern blot analyses were used to evaluate the mRNA for PPARs. Activities of PPARs were assessed using JEG3 choriocarcinoma cells transfected with a PPAR-response element reporter construct (pTK-PPREx3-luc) and treated with PPAR ligands. The PPAR-γ-specific ligand rosiglitazone induced PPAR response element (PPRE)-mediated activity in a concentration-dependent manner, whereas the PPAR-γ-specific irreversible inhibitor GW9662 fully inhibited this induction. However, GW9662 only partially inhibited 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)-induced luciferase activity, suggesting that 15d-PGJ_2 may also activate either of the other isoforms. PPAR-α and -δ are expressed in the amnion, choriodecidua, and placental villous tissues. In the amnion, although for PPAR-α no significant difference in expression was observed with labor, PPAR-δ expression increased significantly ($p < 0.001$). In the choriodecidua, expression of PPAR-α declined with labor ($p < 0.01$), whereas, as in the amnion, PPAR-δ expression increased ($p < 0.05$). In the placenta, both PPAR-α and -δ expression increased with labor ($p < 0.005$). The changes observed with labor suggest that regulation of PPAR expression and function may have roles to the mechanisms that maintain pregnancy or initiate labor.

The initiation of labor is preceded by increased prostaglandin (PG) and proinflammatory cytokine production by gestational tissues. PGD_2 is a major PG in a variety of tissues. It has been found to be elevated in amniotic fluid collected from women undergoing spontaneous labor at term (Berryman et al., 1987). Secretion of PGD_2 by gestational tissues has also been measured and the placenta was found to be a major source of PGD_2 (Mitchell et al., 1982). PGD_2 is readily converted into PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) through a series of dehydration reactions. 15d-PGJ_2, a natural ligand for peroxisome proliferator-activated receptor (PPAR)-γ, has been shown to induce apoptosis in a variety of tissues, including the amnion-derived WISH (Keelan et al., 2001) and JEG3 choriocarcinoma cells (Keelan et al., 1999).

PPARs are members of the nuclear receptor superfamily of transcription factors that control the expression of a large array of genes in a ligand-dependent manner. To date, three human PPAR isoforms have been identified; PPAR-α, PPAR-δ (also known as PPAR-β) and PPAR-γ, the latter being identified as existing in two major subtypes PPAR-γ1 and -γ2). They share common structural features, which include an amino-terminal modulatory domain, a DNA binding domain, and a carboxyl-terminal ligand binding domain (Moras and Gronemeyer, 1998). Each isoform has a distinct tissue distribution, with PPAR-α present in high levels in the kidney, heart, muscle, liver, brown adipose tissue, and gut (Kliewer et al., 1994), whereas PPAR-δ is ubiquitously expressed throughout the body. PPAR-γ is highly expressed in adipose tissue, but it is also present in other tissues, including muscle, liver, heart, adrenal gland, spleen, and placenta (Marvin et al., 2000; Waite et al., 2000).

All three isotypes of PPARs have been shown to modulate lipid metabolism and inflammation, but isotype-specific functions have also been described. PPAR-α, the most clinically

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ABBREVIATIONS: PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; cPGI, carbaprostacyclin; CAT-ELISA, chloramphenicol acetyl transferase-enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PPRE, peroxisome proliferator-activated receptor response element; ANOVA, analysis of variance; COX-2, cyclooxygenase-2; TSL, term spontaneous labor; TNL, term no labor; WY14643, pirinixic acid; GW9662, 2-chloro-5-nitro-*N*-phenyl benzamide; L-165041, 4-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy] phenoxyacetic acid.

relevant mediator of the pharmacological effects of peroxisome proliferators, is a transcription factor that regulates several genes involved in lipid metabolism. It seems to be nonessential for normal reproductive function because mice deficient in PPAR-α seem to exhibit normal fertility (DeLuca et al., 2000). However, it has been proposed to be involved in the development of epidermal barrier in the fetus because WY14643 and ETYA, ligands for PPAR-α, are found to accelerate epidermal barrier development in skin explants from fetal rats in vitro (Hanley et al., 1997).

Activation of PPAR-δ has been shown to increase total plasma cholesterol (Leibowitz et al., 2000) and reverse cholesterol transport (Oliver et al., 2001). It is also shown to be a regulator of embryo implantation. It has been shown that prostacyclin, a natural ligand for PPAR-δ, is a key mediator in the process of implantation and decidualization (Lim et al., 1999). PPAR-δ expression was found to be undetectable in the preimplantation uterus but was strongly induced during the decidualization process (Lim et al., 1999). Further investigation into COX-2-deficient mice show that they exhibit implantation defects which can be corrected with administration of PPAR-δ agonists carbaprostacyclin (cPGI) or L-165041 at the site of implantation (Lim and Dey, 2002). Interestingly, mice deficient in PPAR-δ have developmental defects caused primarily by placental malformation, leading to lethality of over 90% embryos (Barak et al., 2002). This lethality seemed to occur earlier in gestation. These findings confirm that PPAR-δ may play a role in the regulation of embryo implantation.

PPAR-γ is a key regulator of adipogenesis. It has also been shown to regulate differentiation, cell proliferation, and cell death. In gestational tissues, it has been shown to be essential for normal placental development and trophoblast differentiation and invasion (Barak et al., 1999; Tarrade et al., 2001). Ligands for PPAR-γ include the antidiabetic thiazolidinedione drug rosiglitazone, as well as its endogenous ligand 15d-PGJ_2. We have previously reported that PPAR-γ mRNA is expressed in the amnion, choriodecidua, and placenta membranes of gestational tissues collected at term (Marvin et al., 2000). The levels of PPAR-γ expression remained unchanged with labor compared with the nonlabor group. The studies presented here investigated whether PPAR-α and -δ subtypes are expressed in gestational tissues and whether levels change with labor. We also investigated the activity of PPARs by using PPAR agonists; rosiglitazone, 15d-PGJ_2, WY14643, and cPGI and the PPAR-γ antagonist GW9662, in the trophoblastic model cell line JEG3 to better define contributions of PPAR isoforms in this model system.

Materials and Methods

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Ham's F-12/Dulbecco's modified Eagle's medium was obtained from Irvine Scientific (Santa Ana, CA); penicillin-streptomycin-glutamine, and trypsin-EDTA were purchased from Invitrogen (Auckland, New Zealand). 15d-PGJ_2, ciglitazone, rosiglitazone, WY14643, cPGI, PPAR-α, and PPAR-δ probes were purchased from Cayman Chemical (Ann Arbor, MI). GW9662 was a generous gift from Tim Willson (GlaxoSmithKline, Uxbridge, Middlesex, UK). Chloramphenicol acetyl transferase (CAT)-ELISA and dithiothreitol were purchased from Roche Diagnostics (Auckland, New Zealand). Luciferase assay reagent and nitrocellulose membranes were purchased from Promega (Madison, WI) and Amersham Biosciences (Auckland, New Zealand), respectively.

Collection of Tissues. Written informed consent for the collection of human placental tissues was obtained from all women according to the local Human Ethics Committee of the Health Authority of New Zealand. Placentae were obtained after elective caesarean section before the onset of labor (indications: previous caesarean or malpresentation) (TNL; $n = 15$) or after spontaneous labor and uncomplicated vaginal delivery (TSL; $n = 15$) at term (37–42 weeks). The average age of gestation in both groups was 38.6 and 39.4 weeks for TNL and TSL, respectively. Pregnancies complicated by preeclampsia, multiple pregnancy, or birth weight less than 10th percentile were excluded. Reflected amnion was stripped from the choriodecidua, and sections of each (~2 cm^2) were cut, washed briefly in ice-cold phosphate-buffered saline (12 mM Na_2HPO_4, 3.9 mM KH_2PO_4, and 145 mM NaCl) to remove blood, and snap-frozen in liquid nitrogen within 1 h of delivery. Villous placental tissue (~1 g) was also sampled from each placenta and similarly washed and frozen.

Cell Culture. JEG3 choriocarcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in Ham's/F-12: Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin-glutamine at 37°C in 95% air, 5% CO_2. Cells were plated at a density of 300,000 cells/ml in 96-well and six-well plates (Nalge Nunc International, Roskilde, Denmark) for MTT assay and transfection experiments, respectively.

MTT Assay. Mitochondrial activity, as an index of cell viability, was measured using the MTT method (Mosmann, 1983). Briefly, cells were treated in triplicates in 96-well plate. After completion of the experimental period, 20 μl of MTT reagent (5 mg/ml) was added per well of culture (containing 100-μl treatment media). The reaction was allowed to proceed for 4 to 6 h at 37°C. SDS (10%) was then added (200 μl/well) overnight and the absorption at 595 nm measured with a SpectraMax UV plate reader (Molecular Devices Corp., Sunnyvale, CA).

Northern Blot Analysis. RNA extraction, electrophoresis, and Northern blotting analysis for PPAR isoform expression was performed as described previously (Marvin et al., 2000), except that PPAR-α and -δ probes were used. Detection of blots was performed using a Storm 860 PhosphorImager (Amersham Biosciences, Piscataway, NJ), and the results were quantitated using ImageQuaNT (Amersham Biosciences).

Promoter-Reporter Assays. The PPRE-driven luciferase reporter plasmid (pTK-PPREx3-luc) (Forman et al., 1995), PPRE lacking (pTK-luc) and β-actin promoter-driven CAT constructs (pβ actin-CAT) were introduced into JEG3 cells by transfection with FuGENE 6 (Roche Diagnostics, Auckland, New Zealand) as described previously (Marvin et al., 2000). In brief, transfection mixes containing 3 μl of FuGENE 6, 0.5 μg of luciferase construct, and 0.5 μg of pβactin-CAT reporter constructs were transfected into JEG3 cells as per the manufacturer's instructions. After 24 h, media were exchanged with treatment media containing the specified concentrations of 15d-PGJ_2, rosiglitazone, GW9662, WY14643, and cPGI. After another 24 h, cell extracts were prepared using CAT-ELISA lysis buffer (Roche Diagnostics) supplemented with 5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride. CAT and luciferase activity were assayed by CAT-ELISA and luciferase assay reagent (Promega) by using a Spectra Max 250 plate reader (Molecular Devices Corp.) and a 1250 MicroBeta TriLux Jet (PerkinElmer Wallac, Turku, Finland) injecting microplate counter, respectively.

Statistical Analyses. Data were analyzed using the Mann-Whitney U test for tissue expression studies and ANOVA with post hoc Dunnett's test for the cell viability and transfection experiments. A value for $p < 0.05$ (*), < 0.01 (**), and < 0.001 (***) was considered significant.

Results

We have previously reported that 15d-PGJ_2, a natural ligand for PPAR-γ, positively regulates luciferase activity of pTK-PPREx3-luc-transfected JEG3 cells in a concentration-dependent manner (Marvin et al., 2000). It has since been reported that 15d-PGJ_2 has PPAR-γ-dependent and -independent effects (Straus et al., 2000). To investigate the PPAR-mediated or nonmediated effects of 15d-PGJ_2, a PPAR-γ specific irreversible inhibitor, GW9662, was used. As a positive control, JEG3 cells transfected with pTK-PPREx3-luc were treated with rosiglitazone (0–10 μM), a pharmacological ligand for PPAR-γ and ligand-induced luciferase activity was measured. Figure 1 shows that rosiglitazone significantly induced luciferase expression in a concentration-dependent manner with an EC_{50} of 0.3 ± 0.08 μM that is comparable with values reported for PPAR-γ (Forman et al., 1995; Lehmann et al., 1995). At 10 μM, rosiglitazone stimulated a 9.4 ± 0.5-fold (mean ± S.E.M.) increase in luciferase activity and this effect was completely inhibited by GW9662 (10 μM) (Fig. 2). However, 15d-PGJ_2-induced luciferase activity was only partially inhibited by GW9662 from 11.1 ± 0.1 down to 7.6 ± 0.1-fold increase in 15d-PGJ_2-induced luciferase activity, suggesting that the effects of 15d-PGJ_2 may contribute, in part, to the activation of other PPAR isoforms. To confirm the activity of PPAR-α and -δ in JEG3 cells, transfected cells were also treated with WY14643 (100 μM) and cPGI (1 μM) and both ligands induced a 1.9 ± 0.6- and 2.4 ± 0.5-fold increase in PPRE-mediated luciferase activity, respectively (data not shown).

The functionality of the PPAR ligands were further investigated by assessing the effect of rosiglitazone, 15d-PGJ_2, WY14643, cPGI, and GW9662 on cell viability by using the MTT method (Mosmann, 1983). Concentration-response experiments were performed (data not shown), and 15d-PGJ_2 and WY14643 were the only ligands that significantly altered cell viability. Figure 3 shows the effect of rosiglitazone (10 μM), 15d-PGJ_2 (10 μM), WY14643 (100 μM), cPGI (1 μM), and GW9662 (10 μM) on JEG3 cell viability. Rosiglitazone, cPGI, or GW9662 did not significantly alter cell viability. However, 15d-PGJ_2 and WY14643 reduced cell viability to 37.56 ± 11.5 and 61.91 ± 7.9% of control (mean % control ± S.E.M.), respectively (Fig. 3).

The presence of PPAR-α and -δ were assessed in intrauterine tissues by Northern blot analysis. We have previously shown that PPAR-γ is expressed in amnion, choriodecidua, and placenta membranes of intrauterine tissues, and its expression remained unchanged with labor (Marvin et al., 2000). We now show that PPAR-α and -δ are also expressed in the amnion, choriodecidua, and placenta from gestational tissues collected after spontaneous (TSL) and caesarean (TNL) delivery at term (Fig. 4). PPAR expression was quantitated and normalized to 28S rRNA and the median densitometry values and interquartile ranges are summarized in Table 1. The expression of PPAR-α was variable between all three tissues collected from labor (TSL) and nonlabor (TNL) group. In the amnion, PPAR-α expression was unchanged between TSL and TNL. In contrast, its expression was much reduced with labor in the choriodecidua membrane ($p < 0.01$) but was significantly increased in the placenta villous tissue ($p < 0.001$). The expression of PPAR-δ was higher ($p < 0.001$) in all tissues collected from TSL group compared with TNL. Expression of PPAR-α and -δ were also measured in gestational cell lines, JEG3 cells and WISH cells. Both PPAR-α and -δ are expressed in JEG3 cells in either forskolin-treated or untreated control cells. However, PPAR-δ but not PPAR-α is expressed in WISH cells.

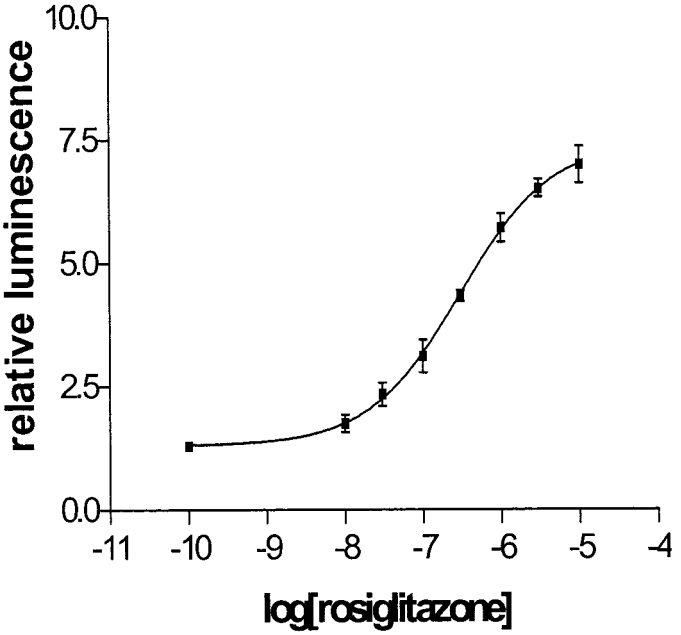

Fig. 1. Dependence of luciferase reporter activity on rosiglitazone (0–10 μM). Data for luciferase activity in pTK-PPREx3-luc-transfected cells have been normalized to CAT [luminescence counts per second (LCPS) per picogram of CAT] and expressed relative to the same value for similarly treated cells which had been transfected with pTK-luc. Relative luminescence = $(LCPS/pg\ CAT)_{pTK\text{-}PPREx3\text{-}luc}$/ $(LCPS/pg\ CAT)_{pTk\text{-}luc}$. The data represent two experiments performed in triplicate for each construct and treatment condition ($p < 0.01$ was considered significant after ANOVA).

Discussion

We have previously demonstrated that PPAR-γ mRNA is expressed in the amnion, choriodecidua, and villous placental

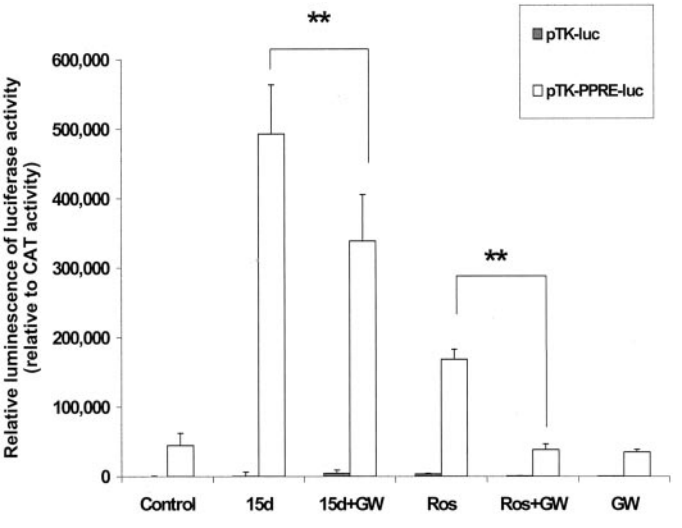

Fig. 2. Effect of 15d-PGJ_2 (10 μM) and rosiglitazone (10 μM) on induction of pTK-PPREx3-luc luciferase reporter activity in the absence and presence of a PPAR-γ irreversible inhibitor, GW9662 (10 μM). The data represent three experiments performed in triplicate. A **, $p < 0.01$ was considered significant after ANOVA with post hoc Dunnett's test.

tissues collected at term and that its presence is not significantly different between labor (TSL) and nonlabor (TNL) group (Marvin et al., 2000). The present study demonstrates that PPAR-α and -δ mRNA are also expressed in all three gestational tissues examined. The trophoblastic cell line JEG3 is functionally responsive to the PPAR ligands rosiglitazone, 15d-PGJ_2, WY14643, and cPGI. Similar to PPAR-γ, the expression of PPAR-α was unchanged in the amnion. However, its expression was reduced in the choriodecidua and higher in the placental tissues collected at term, with labor. This suggests that PPAR-α may play a role in regulation of the chorion membrane and/or decidua.

PPAR-α is expressed most abundantly in highly oxidative tissues where it plays a key role in regulation of cellular uptake, activation, and β-oxidation of fatty acids. The role of PPAR-α in pregnancy is unclear. However, mice deficient in PPAR-α have normal fertility, suggesting that PPAR-α may not be essential for normal reproductive function in mice. Activation of PPAR-α has also been shown to exert anti-inflammatory effects such as inhibition of IL-1β-induced production of IL-6, PG, and expression of COX-2 by smooth muscle cells (Staels et al., 1998). It has been proposed that it may do this through inhibition of the NF-κB signaling pathway (Chinetti et al., 1998). PPAR-α may exert anti-inflammatory properties and hence may potentially inhibit processes promoting labor in the gestational tissues.

The expression of PPAR-δ is increased in all three tissues examined, with labor (Fig. 4). Little is known about the regulatory role of PPAR-δ later in gestation. However, it was found to be expressed in implantation sites within the uterus (Lim et al., 1999) and has been shown to play a role in implantation. Barak et al. (2002) reported that PPAR-δ deficiency is lethal to over 90% of embryos. This lethality seemed to occur earlier during gestation, with PPAR-δ null embryos dying in parallel to the appearance of an abnormal gap in the placentodecidual interface (Barak et al., 2002). Treatment with PPAR-δ ligands, cPGI, or L-165041 has been shown to restore implantation defects in COX-2 null mice (Lim et al., 1999). This supports the conclusion that PPAR-δ may play a role in maintaining reproductive capacity in females.

The PPAR-γ ligand 15d-PGJ_2 has been shown to have both PPAR-γ-dependent and -independent activities. We have previously shown that 15d-PGJ_2 induced apoptosis in JEG3

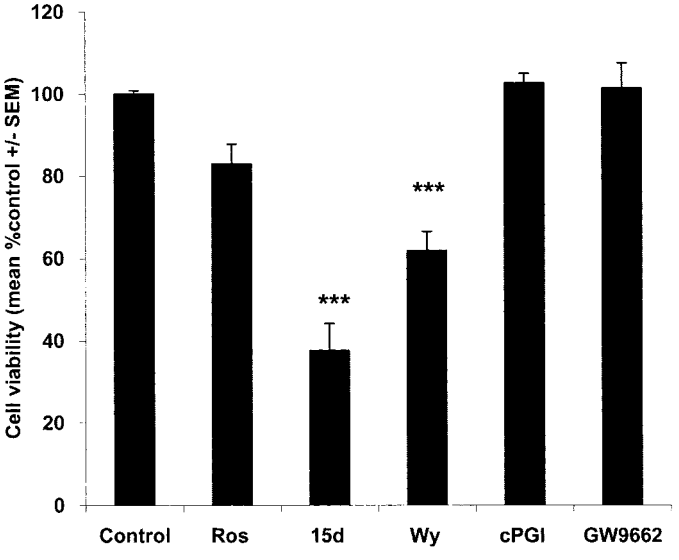

Fig. 3. Effect of PPAR ligands rosiglitazone (10 μM), 15d-PGJ_2 (10 μM), WY14643 (100 μM), cPGI (1 μM), and GW9662 (10 μM) on cell viability as measured by MTT assay. The data represent three experiments performed in triplicate. ***, $p < 0.001$ was considered significant after ANOVA with post hoc Dunnett's test.

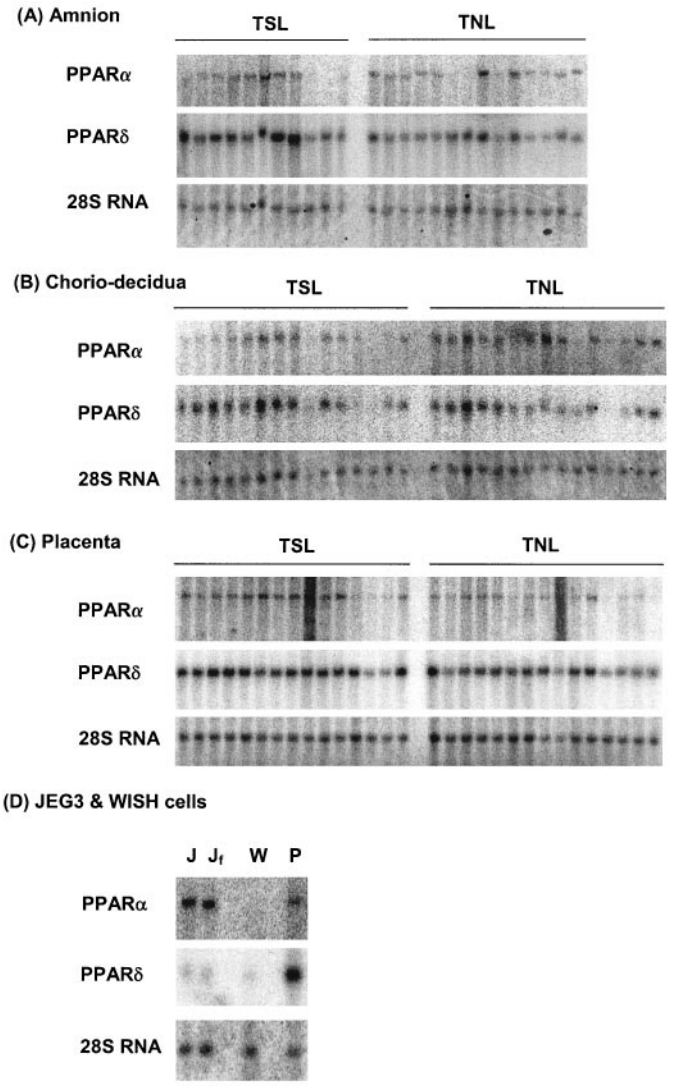

Fig. 4. Northern blot showing expression PPAR-α and -δ in gestational tissues: amnion (A), choriodecidua (B), and placenta (C) collected at term after spontaneous labor (TSL) or Caesarean section (TNL) and in D, untreated (J) and forskolin-treated (J_f) JEG3, WISH (W) cells, and placenta (P).

TABLE 1

Relative expression (median and interquartile range) of PPAR-α and -δ mRNA expression in intrauterine tissues

Data are relative densitometry values normalized to 28 S RNA from all samples shown in Fig. 4.

	TSL	TNL	
PPAR-α			
Amnion	0.943(0.554,1.103)	0.766(0.480,0.983)	N.S.
Choriodecidua	0.563(0.353,0.763)	0.960(0.846,1.230)	$p < 0.01$
Placenta	0.801(0.685,1.131)	0.404(0.309,0.603)	$p < 0.01$
PPAR-δ			
Amnion	0.370(0.288,0.605)	0.212(0.165,0.284)	$p < 0.01$
Choriodecidua	1.374(1.181,1.527)	1.078(0.701,1.317)	$p < 0.05$
Placenta	2.265(2.080,2.508)	1.716(1.529,2.060)	$p < 0.01$

NS, not significant.

(Keelan et al., 1999) and WISH cells (Keelan et al., 2001) and that it activates PPRE in a concentration-dependent manner (Marvin et al., 2000). We now show that 15d-PGJ_2-induced PPRE activation can be partially inhibited by GW9662, an irreversible inhibitor of PPAR-γ. In contrast, GW9662 completely inhibited rosiglitazone-induced PPRE activation. These results indicated that although 15d-PGJ_2 exerts some of its actions via activation of PPAR-γ, clearly there are alternate mechanisms involved in its biological actions. Among these seem to be PPAR-α- and/or -δ-dependent pathways, resulting in a proportion of PPRE-mediated transcription that is independent of PPAR-γ.

The exact roles that PPAR-α and -δ play in the mechanisms that initiate labor remain unclear. However, results presented in this study suggest that they are regulated in the setting of labor. At present, it is also unclear how cytokines regulate PPAR expression. However, studies conducted in our laboratory have demonstrated that there is a complex interaction of both positive and negative feedback cytokine loops that directly effect PG production (Sato et al., 2003). Preliminary studies in our laboratory have demonstrated regulation of PPARs, particularly PPAR-γ, where suppression of PPAR-γ resulted in suppression of uterotonic PG production by gestational tissues. Further studies will need to be conducted to determine how PPAR expression is involved in or interacts with the cytokine-PG balance either maintaining pregnancy or initiating labor. In summary, the regulation of PPAR expression and function may contribute to the mechanisms that initiate labor. A better understanding of the regulation of PPARs may provide insights to the mechanisms that initiate labor.

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